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D-Cycloserine facilitates fear extinction in adolescent rats and differentially affects medial and lateral prefrontal cortex activation

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ABSTRACT

Adolescent humans and rodents are impaired in extinguishing learned fear relative to younger and older groups. This impairment could be due to differences in recruitment of medial prefrontal cortex (PFC), orbitofrontal cortex (OFC), or amygdala during extinction. For example, unlike juveniles and adults, adolescent rats do not express extinction-induced increases in phosphorylated mitogen activated protein kinase (pMAPK), a marker of synaptic plasticity, in the medial PFC. The NMDA receptor partial agonist D-cycloserine (DCS) improves extinction retention in adolescent rats. We investigated whether DCS affected recruitment of the PFC and amygdala during extinction by measuring pMAPK-immunoreactive (IR) neurons. Adolescent rats were trained to fear a conditioned stimulus in one context followed by extinction in a second context or equivalent context exposure only (i.e., no extinction). DCS (15 mg/kg, s.c.) or saline was administered systemically immediately after extinction training or context exposure. DCS enhanced extinction learning and this was associated with increased activation of the MAPK signaling pathway in the OFC after extinction training and increased activation in the medial PFC and amygdala at extinction retention. These findings suggest that DCS improves extinction learning in adolescents because it augments OFC contributions to extinction learning, enabling better medial prefrontal contributions to extinction retention.

1. Introduction

Adolescent humans and rodents are impaired in extinguishing learned fear compared to younger and older groups (Baker and Richardson, 2015; Kim et al., 2011; McCallum et al., 2010; Pattwell et al., 2011). This impairment might interfere with the long-term effectiveness of exposure-based therapy, the current first-line treatment for anxiety disorders, because this therapy is based on the principles of extinction (e.g., Graham and Milad, 2011; McNally, 2007; Vervliet et al., 2013). Although anxiety disorders often manifest in late childhood/early adolescence (e.g., Kessler et al., 2012; Kim-Cohen et al., 2003) and disorders that emerge before adulthood are recognized as being both more difficult to treat and more costly (Lee et al., 2014), most adolescents and adults (i.e., 50–60%) achieve reductions in symptoms during cognitive behavioral therapies involving exposure (Loerinc et al., 2015; Piacentini et al., 2014; Warwick et al., 2016). Unfortunately, a higher proportion of youth (~40%) relapse after successful exposure-based therapies for paediatric anxiety (Ginsburg et al., 2014) compared to adults (23%) (Dimauro et al., 2013), indicating that long-term treatment outcomes for adolescents with anxiety disorders can be improved. Towards this goal, one focus of preclinical research has been to identify pharmacological adjuncts that enhance extinction in adolescents. One of the most rapidly translated adjuncts from preclinical studies to humans is the NMDA receptor partial agonist D-cycloserine (DCS). DCS facilitates extinction retention in adolescent (McCallum et al., 2010) and adult rats (Ledgerwood et al., 2003, 2004; Walker et al., 2002), as well as exposure-therapy in clinically anxious samples (e.g., Guastella et al., 2008; Otto et al., 2010; Ressler et al., 2004).

Nonetheless, the cause of the deficits in extinction during adolescence and the mechanisms through which DCS may overcome these remain poorly understood. The prefrontal cortex (PFC) and basolateral amygdala (BLA) are critical for inhibiting fear via extinction (Sotres-Bayon et al., 2009), and there is evidence that adolescents may differ in recruitment of both during extinction learning and/or retention. For example, adolescents do not exhibit extinction-induced increases in expression of activity-dependent markers of synaptic plasticity (phosphorylated mitogen activated kinase; pMAPK) in the medial PFC after extinction training (Baker and Richardson, 2015; Kim et al., 2011) or neuronal activation (c-Fos) at extinction retention (Pattwell et al., 2012). There is also evidence that extinction-induced increases in pMAPK activation are not observed in the BLA as well (Baker and

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Richardson, 2015). Given that NMDA receptors are critical for extinction, it has been suggested that a lack of NMDA receptor activation contributes to inefficient prefrontal-amygdala recruitment during extinction in adolescents and so impairs extinction learning at this developmental stage (Baker et al., 2016; Baker and Richardson, 2017; Casey et al., 2015). It follows that administration of DCS may augment extinction learning in adolescent rats because it facilitates prefrontal activity during extinction training or test. Indeed, findings from one study lend support for this idea. Gupta et al. (2013) reported that systemic DCS up-regulated pMAPK expression in the IL, PL, and lateral (but not medial) subdivision of the central amygdala (CeA), and down-regulated pMAPK expression in the basal amygdala, 6 h after extinction training in juvenile rats (24–28 days old). However, it is unknown whether a similar effect occurs in adolescent rats.

Here we addressed this issue. We used expression of pMAPK to investigate whether the facilitation of extinction by DCS in adolescent rats is associated with altered recruitment of medial PFC (prelimbic [PL]; infralimbic [IL]), lateral PFC (orbitofrontal cortex [OFC]; rostral agranular insular cortex [RAIC]), and amygdala (basolateral nucleus; central nucleus). The effects of DCS on pMAPK expression in the PFC and amygdala were examined in adolescent rats that were experimentally naive (Experiment 1), which had undergone extinction training (Experiment 2), and which were tested for extinction retention (Experiment 3).

2. Materials and methods

2.1. Animals

Subjects were experimentally naive male adolescent Sprague-Dawley rats ($N = 61$) obtained from the breeding colony maintained by the School of Psychology at UNSW Sydney. The animal housing conditions were the same as in our previous studies (Baker et al., 2013; Baker and Richardson, 2015). Rats were weaned at P21–24 and randomly assigned to groups with no more than one rat per litter being assigned to any given group. Across the three experiments, rats were P34–37 when injected with DCS or saline, and the animals were either naive to any experimental procedures (Experiment 1) or had received Pavlovian conditioning and extinction (Experiments 2 and 3). All procedures were approved by the Animal Care and Ethics Committee at UNSW and conducted in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (8th Edition, 2013).

2.2. Experimental procedure

The apparatus and behavioral procedures were similar to those previously used in our laboratory for studies on extinction in adolescent rats (Baker et al., 2013; Baker and Richardson, 2015; Kim et al., 2011; McCallum et al., 2010). In Experiment 1, rats received handling for 3–4 min each day on the two days prior to injection of saline or DCS to acclimatize to the experimenter. In Experiments 2 and 3, rats received similar handling each day on the two days prior to conditioning and then, on each day, animals were placed in the conditioning chamber for 8–10 min. Fear acquisition occurred in one set of Med Associates chambers (Context A) while extinction and test were conducted in a second set of chambers (Context B), which had different visual features, flooring, and lighting to Context A. This practice maximizes the detection of freezing elicited by the CS, rather than the context, at test. Context A had a grid floor with a tray of corncob bedding underneath and was unilluminated. Context B had a clear Perspex sheet over the grid floor and the tray did not contain any bedding. The Context B chambers also had vertical black and white striped paper attached to the exterior of the ceiling and door, and a white lamp above the chamber provided illumination (~4 lx inside the chambers) for this context. The CS was a 10 s white-noise delivered through a speaker in the chamber; noise levels increased by 4 dB above background when the CS was presented. The US was a scrambled 0.6 mA, 1 s footshock delivered through the grid-floor. Presentations of the CS and US were controlled by a computer running Med-PC IV software (Med Associates). The chambers were cleaned with tap water after each experimental session.

Pavlovian conditioning, extinction, and test occurred on consecutive days approximately 24 h apart. There was a 2 min adaptation period at the beginning of each session. During conditioning rats received three CS trials co-terminating with the US. The intertrial intervals were 135 and 85 s (mean of 120 s). Extinction training consisted of 30 non-reinforced presentations of the CS (10 s each; 10 s intertrial interval). For statistical analyses of within-session extinction, 6 trials were averaged to represent 1 block. In Experiment 2, "No Extinction" control groups received conditioning and were then exposed to the extinction context without any CS presentations for an equivalent duration as extinction training. Animals were tested the day after extinction with a single CS presented for 2 min (in Experiment 3).

D-cycloserine (Sigma C6880; 15 mg/kg), or saline, was administered subcutaneously (in the nape of the neck) in a volume of 1 ml/kg. DCS was freshly dissolved in 0.9% sterile saline (wt/vol) on the day of use and kept on ice. In the first experiment naive rats were injected with DCS or saline. In Experiments 2 and 3, rats were injected with DCS or saline immediately after extinction (i.e., within 2–3 min of the last trial) (McCallum et al., 2010) or an equivalent time in the same context (i.e., groups No Extinction).

2.3. Tissue processing, immunohistochemistry, and neuronal counting

Rats were sacrificed either one or 24 h following DCS or saline injections in Experiment 1. In Experiment 2 rats were sacrificed 1 h after the extinction/context-only exposure session, a time-point when pMAPK levels peak after extinction in limbic regions (Fischer et al., 2007; Herry et al., 2006). In Experiment 3 rats were sacrificed 1 h after the extinction retention test. Rats were deeply anesthetized with sodium pentobarbital (433 mg/kg, i.p., diluted in 1:2 parts saline). The rats were then transcardially perfused with 50 ml of 0.9% saline containing 1% sodium nitrite and heparin (5000 I.U./ml), followed by 150–200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were extracted, postfixed for 1–2 h, washed in 0.1 M PB saline (pH 7.2), and cryoprotected in 20% sucrose in PB saline (overnight or up to 48 h). A rat brain atlas (Paxinos and Watson, 2009) guided the blocking of the brains using a matrix. Four serially adjacent sets of 40 μm coronal sections were cut using a cryostat (CM1950, Leica Microsystems) from each brain and stored in 0.1% sodium azide in 0.1 M PB saline (pH 7.2).

One series of sections was selected from each rat and processed for phospho-p44/42MAPK-immunoreactivity (pMAPK-IR) in combination with tyrosine hydroxylase (TH) using two-color peroxidise immunohistochemistry, with methods previously established for pMAPK-IR (Baker and Richardson, 2015; Kim et al., 2011). In this procedure free-floating sections were washed with gentle agitation in 0.1 M PB (pH 7.4; hereafter referred to as PB), followed by two 30 min washes in 50% ethanol, the second of which contained 3% H_2O_2, and were then incubated for 30 min in 5% normal horse serum (NHS) in PB. Sections were then incubated in rabbit antiserum against pMAPK (1:2000; phospho-p44/42 MAPK (Erk1/2) [Thr202/Tyr204] (D13.14.4E) XP® Rabbit mAb, #4370; Cell Signaling Technology, USA) and sheep antiserum against TH (1:5000; #AB1542; Chemicon® Merck) for 48 h at 4 °C, with gentle agitation. The primary antibody was diluted in 2% NHS in PBT-X, which consisted of PB and 0.2% Triton X-100. After washing off unbound primary antibody with PB with gentle agitation, sections were incubated overnight at room temperature in biotinylated donkey anti-rabbit IgG (1:2000; 711–065-152; Jackson ImmunoResearch Laboratories, USA) diluted in 2% NHS PBT-X. After washing, sections were incubated for 2 h at room temperature in 2% NHS PBT-X combined with avidin–biotin–peroxidase complex (ABC)